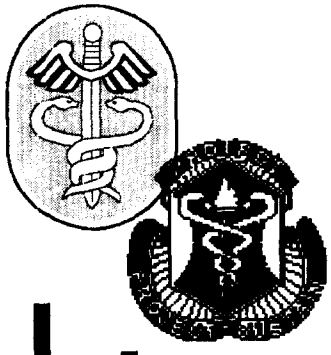

# U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

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# *In Vivo* Characterization of Intracellular Signaling Pathways Activated by the Nerve Agent Sarin

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**14. ABSTRACT**

Organophosphorous (OP) nerve agents, such as sarin, exert acute effects by inhibiting acetylcholinesterase in the central and peripheral nervous systems, which results in accumulation of acetylcholine and, in turn, an excessive stimulation of nicotinic and muscarinic receptors. Preliminary evidence using diverse OPs indicates that the DARPP-32/PP-1 signaling pathway is activated by nicotinic receptor stimulation. We investigated whether treatment of whole animals with sarin activated the DARPP-32/PP-1 signaling cascade. Both a seizure-inducing dose (1.0 x LD50) and a sub-seizure threshold dose (0.5 x LD50) of sarin were tested. Rats receiving the sub-threshold dose were asymptomatic for seizures. A selective increase in phospho-T75 DARPP-32 levels was observed in striatum from these rats 30 min after exposure. Rats displaying seizure activity following administration of a 1.0 x LD50 dose of sarin displayed changes in several phosphoproteins, including T75 DARPP-32. Transient increases in T75 DARPP-32, T183 ERK, and S133 CREB phosphorylation were followed by reductions in phosphorylation at three DARPP-32 sites (T34, S102, and S137) and reductions in S94 spinophilin and S897 NR1. Another glutamate receptor site, S845 GluR1, was unaffected by sarin treatment. This study represents the first comprehensive analysis of signal transduction pathways activated in response to sarin exposure in whole animals.

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## ABSTRACT

Organophosphorous (OP) nerve agents, such as sarin, exert acute effects by inhibiting the enzyme acetylcholinesterase in the central and peripheral nervous systems. Inhibition of acetylcholinesterase results in accumulation of acetylcholine and, in turn, an excessive stimulation of nicotinic and muscarinic receptors. Preliminary evidence using diverse OP compounds indicates that the DARPP-32/PP-1 signaling pathway is activated by nicotinic receptor stimulation. This study was to investigate whether treatment of whole animals with sarin would activate the DARPP-32/PP-1 signaling cascade as predicted in our pilot studies. Both a seizure-inducing dose (1.0 x $LD_{50}$) and a sub-seizure threshold dose (0.5 x $LD_{50}$) of the nerve agent were tested. Rats receiving a sub-threshold dose of sarin (0.5 x $LD_{50}$) were asymptomatic for seizures. A selective increase in phospho-T75 DARPP-32 levels was observed in striatum from these rats 30 min after exposure. Rats displaying seizure activity following administration of a 1.0 x $LD_{50}$ dose of sarin displayed changes in several phosphoproteins, including T75 DARPP-32. Transient increases in T75 DARPP-32, T183 ERK, and S133 CREB phosphorylation were followed by reductions in phosphorylation at three DARPP-32 sites (T34, S102, and S137) as well as reductions in S94 spinophilin and S897 NR1. Another glutamate receptor site, S845 GluR1, was unaffected by sarin treatment. This study represents the first comprehensive analysis of signal transduction pathways activated in response to sarin exposure in whole animals.

## INNOVATION

An opportunity presented itself for this collaborative project by investigators from two distinguished laboratories. The senior scientists at the U. S. Army Medical Research Institute of Chemical Defense (USAMRICD) have more than 50 years of combined research experience on the neuropharmacological and neurochemical mechanisms and behavioral consequences of chemical warfare nerve agents. Their laboratories are equipped with a head-focused microwave irradiation device and unique nerve agent exposure facility. The senior scientists at Intra-Cellular Therapies, Inc. (ITI), on the other hand, are well-experienced in the intracellular pathways that fine-tune the activity of kinases, enzymes that attach phosphates onto target proteins, and phosphatases, which carry out the reverse process and remove the phosphates.

This study was also unique in that it would characterize the effects of nerve agents on intracellular signaling pathways altered *in vivo*. This was made possible by the use of 1) a specially designed microwave to arrest alterations of phosphorylation state *in vivo* after nerve agent exposure and 2) phospho-specific antibodies that have been developed to specifically monitor changes in phosphorylation. These experiments provided a "snapshot" of how nerve agents alter the neurotransmitter signaling pathways in the brain.

## TRANSITION OF RESEARCH

Results from this project will lay the groundwork for additional studies to characterize the changes in protein phosphorylation resulting from 1) acute exposure to other nerve agents, 2) seizures induced by nerve agents, and 3) low level acute and chronic exposure to various nerve agents.

## MILITARY RELEVANCE

These studies have a major potential to result in the development of novel pharmaceuticals and/or diagnostics that may have several uses to the United States military. An understanding of the acute effects of nerve agents on brain function will aid in the development of protective drugs with better central nervous system efficacy than drugs such as atropine and diazepam, which are currently available. For instance, we anticipate the development of compounds that are more effective in negating convulsant effects of exposure to organophosphorus anticholinesterase agents. Moreover, an understanding of the intracellular pathways altered by high-dose or chronic low-dose stimulation of cholinergic receptors is likely to lead to the development of pharmaceuticals that reverse the cognitive deficits associated with nerve agent exposure, extend normal cognitive performance in environments contaminated with chemical agents, and may provide means of increasing cognitive performance in deployment environments. Finally, an understanding of the long-term effects of nerve agent exposure as distinguished from the response to stress and fear present in combat deployments may lead to the development of diagnostics that are effective in distinguishing between, and perhaps treating the ill effects of, chemical agents and stress physiology. Such diagnostic agents would be useful in monitoring the health of military personnel in ways that are not presently possible.

# INTRODUCTION

A well-characterized mediator of the biochemical, electrophysiological, transcriptional and behavioral effects of several major brain neurotransmitters is a phosphoprotein known by the acronym DARPP-32 (dopamine (DA) and cAMP-regulated phosphoprotein of molecular weight 32KDa). In its phosphorylated but not dephosphrylated form, DARPP-32 is an extremely potent inhibitor of protein phosphatase-1 (PP-1), a major multifunctional serine/threonine protein phosphatase in the brain. PP-1, in turn, regulates the phosphorylation state and activity of many downstream physiological effectors, including various neurotransmitter receptors and voltage-gated ion channels. Studies have shown that the DARPP-32/PP-1 cascade is a major target for psychostimulants and antischizophrenic drugs (see reviews by Greengard et al., 1999; Fienberg and Greengard, 2000; Greengard, 2001).

DARPP-32 is highly enriched in prefrontal cortex and striatum. Activation of the D1-subclass of DA receptors, leading to stimulation cAMP-dependent protein kinase (PKA), results in phosphorylation of DARPP-32 at Thr-34 (T34), thereby converting DARPP-32 into a potent inhibitor of PP-1 (Hemmings et al., 1984a). This effect is counteracted by activation at the D2-subclass of DA receptors, which results in (a) inhibition of PKA and (b) stimulation of the $Ca^{+2}$/calmodulin-dependent protein phosphatase signaling cascade, which dephosphorylates T34-DARPP-32 (Nishi et al., 1999). Activation of the D1-subclass of DA receptors also decreases phosphorylation of Thr-75 (T75)-DARPP-32, which reduces inhibition of PKA and, thereby, facilitates signal transduction by means of the PKA/T34-DARPP-32/PP-1 signaling cascade (Bibb et al., 1999). The efficacy of this signaling cascade is also regulated by the phosphorylation state of DARPP-32 at Ser-102 (S102) and Ser-137 (S137) (see Figure 1). For example, S102 on DARPP-32 is phosphorylated by casein kinase II (CK2). Increases in phosphorylation at site S102 increase the efficiency of phosphorylation at the T34 site of phosphorylation by PKA but not by cGMP-dependent protein kinase (PKG) (Girault et al., 1989). DARPP-32 is also phosphorylated on amino acid S137 by casein kinase I (CK1). Increases in phosphorylation at this site decrease the rate of dephosphorylation by protein phosphatase 2B (PP-2B) at the T34 site. The physiological effect of phosphorylation at S102 and S137 is to potentiate signaling through the dopamine/D1/PKA/DARPP-32/PP-1 pathway and to reduce signaling through the glutamate/$Ca^{+2}$/PP-2B/DARPP-32/PP-1 pathway. PP-1 controls the state of phosphorylation and activity of numerous physiologically important substrates, including neurotransmitter receptors, voltage-gated ion channels, ion pumps and transcription factors. As a result, neurotransmitters that increase or decrease the phosphorylation state of DARPP-32 inhibit or activate, respectively, PP-1 and, thereby, increase or decrease the state of phosphorylation and activity of a large array of downstream physiological effectors (Greengard et al., 1999).

In addition to DARPP-32, PP-1 also interacts with a distinct group of proteins termed targeting subunits that serve to localize the catalytic subunits of PP-1 to specific subcellular compartments. One of these proteins is spinophilin, which has been demonstrated to be an actin binding protein (Hsieh-Wilson et al., 2003). Knockout of spinophilin has shown that spinophilin modulates excitatory synaptic transmission and dendritic spine morphology. Spinophilin knockout mice were also resistant to kainite-induced seizure (Feng et al., 2000). Spinophilin is

phosphorylated by PKA at Ser-94 (S94) and Ser-177. Phosphorylation at these sites modulates the association of spinophilin and the actin cytoskeleton.

Both N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are ionotropic glutamate receptors that mediate major excitatory neurotransmission. AMPA receptors mediate the majority of fast excitatory synaptic transmission, while NMDA receptors play an essential role in the modulation of excitatory synaptic transmission due to their permeability to calcium ions and ability to activate downstream calcium-dependent signal transduction processes. Phosphorylation of the AMPA receptor at Ser845 (S845) increases the apparent open-channel probability of the receptor (Roche et al., 1996) and appears to be necessary for synaptic plasticity and cognitive functions related to learning and memory, such as long-term potentiation and long-term depression (Lee et al., 2003). Ser897 (S897) is a PKA-dependent site on subunit 1 of the NMDA-responsive type glutamate receptor (NR1) that is involved in regulating NMDA receptor conductance. Extracellular signal-regulated protein kinase (ERK) and cAMP response element binding protein (CREB) are transcription factors that are likely to be involved in learning and memory-related physiological processes. Phosphorylation of these proteins has been previously demonstrated to play a role in regulating their function.

The DARPP-32/PP-1 cascade is responsive to a large number of neurotransmitters in addition to DA (Hemmings et al., 1984b; Walaas and Greengard, 1984). These include glutamate (Halpain et al., 1990), γ-aminobutyric acid (GABA) (Snyder et al., 1994), adenosine (Svenningsson et al., 1998), cholecystokinin (CCK) (Snyder et al., 1994), neurotensin (Girault et al., 1989) and others (Tsou et al., 1993). Direct proof for the role of the DARPP-32/PP-1 signaling cascade in mediating the actions of these various first messengers has come from both *in vitro* manipulations using injection of kinase and phosphatase molecules and from gene knockout experiments. The latter type of experiment using the DARPP-32 knockout has shown that DARPP-32 is essential to the action of various neurotransmitters and that the DARPP-32/PP-1 cascade modulates the phosphorylation state of several important ligand- and voltage-gated ion channels. These include the NMDA-type glutamate receptors (Fienberg et al., 1998) and the AMPA-type glutamate receptors (Yan et al., 1999).

A seizure-inducing dose of sarin would be expected to result in high levels of acetylcholine, the endogenous agonist that acts at both muscarinic and nicotinic receptors, and such cholinergic stimulation leads to increased dopamine activity as reflected by increases in dopamine metabolites seen in striatum following convulsant doses of the nerve agent soman (Shih, 1982; Shih and McDonough, 1997). That DA is involved in the etiology of nerve agent-induced seizures is also supported by the finding that the D1 receptor antagonist SCH23390 will block seizure activity produced by the nerve agent soman (Bourne et al., 2001). Interestingly, Blockade of the D2 receptor with sulpiride augmented the evoked seizure activity by soman (Bourne et al., 2001).

A direct interaction of the cholinergic system with the DARPP-32/PP-1 cascade has not yet been reported. Preliminary results demonstrate that treatment of neostriatal slices with nicotine (100 μM), a cholinergic acetylcholine receptor agonist, stimulated DARPP-32 phosphorylation on T34 by approximately 7-fold at 15 and 30 seconds of incubation. The effect of nicotine on

T34 phosphorylation was abolished by a dopamine D1 antagonist, SCH23390, or by the combination of SCH23390 and the dopamine D2 antagonist raclopride. These results suggest that the effect of nicotine is mediated through the release of DA. Additional experiments have generated data that lead to the following model. At low concentrations of nicotine, direct activation of nicotinic receptors on dopaminergic nerve terminals leads to DA release and resultant activation of post-synaptic dopamine D2 receptors leading to dephosphorylation of DARPP-32. Conversely, at high concentrations of nicotine, glutamate activation of DA release leading to activation of dopamine D1 receptors and phosphorylation of DARPP-32 is dominant.

The effects of organophosphorus cholinesterase inhibitors, such as insecticides and chemical warfare nerve agents, on the intracellular signaling pathways have not yet been investigated. Therefore, the objective of this project was to document and identify for the first time changes in phosphorylation state of selected striatal phosphoproteins that are associated with exposure of rats to the organophosphorus nerve agent sarin. Both seizure-inducing doses (i.e., 1.0 x $LD_{50}$) and sub-seizure threshold doses (i.e., 0.5 x $LD_{50}$) of the nerve agent were tested to determine whether certain of these markers are predictive of sarin exposure in non-symptomatic animals.

Monitoring protein phosphorylation *in vivo* in rodents requires the utilization of two non-standard techniques. Both are now available. The first is a method of sacrificing the rodent so that protein phosphorylation changes can be preserved. The second technique involves the use of antibodies that are developed to specifically recognize the phosphorylated form of a given protein and not the dephosphorylated form.

1. *Preserving protein phosphorylation changes in vivo*: Phosphoproteins like DARPP-32 are subject to the effects of proteases and phosphatases. This is a special problem for studies in intact animals, which are aimed at estimating the levels of phosphorylated proteins present *in vivo*. To carry out these studies it is necessary to rapidly inactivate proteases and phosphatases at the time of death so as to preserve the phosphorylated state of the protein. The sacrifice of rodents by microwave irradiation (Guidotti et al., 1974) effectively preserves phosphorylated proteins from the postmortem activity of phosphatases and proteases. Microwave irradiation devices designed to focus on the head region of small rodents (i.e., mouse, rat) are currently available for this purpose to study brain phosphoproteins.

2. *Phospho-specific antibodies*: In 1985 Czernick and Greengard developed biochemical methods to label a chemically synthesized peptide *in vitro* and the immunization procedures that facilitate the generation of a phospho-specific antibody (Czernick et al., 1991). These antibodies recognize a specific phosphorylated amino acid in a specific protein and not the dephosphorylated form. Without this technique it would be impossible to monitor phosphorylation changes without the administration of $^{32}P$ to the whole animal with the additional need to immunoprecipitate the labeled protein. Such a procedure would be untenable today due to safety considerations.

## MATERIALS AND METHODS

Animals: A total of 30 male Crl:CD(SD)IGSBR Sprague-Dawley rats, weighing 280-340g at the time of experiment, were used in this study. Animals were obtained from Charles River Labs (Kingston, NY) and housed individually in temperature (21 ± 2 °C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12-h light-dark full spectrum lighting cycle with lights on at 0600 h. Laboratory chow and water were freely available. Experiments were conducted at the U. S. Army Medical Research Institute of Chemical Defense (USAMRICD) and brain samples shipped to Intracellular Therapeutics, Inc. (ITI) for processing. The research environment and protocol for animal experimentation were approved by the institutional animal care and use committee at USAMRICD. Animal facilities at USAMRICD are accredited by AAALAC.

Materials: Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U. S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 0.5 ml/kg subcutaneously (s.c.).

BCA protein assay kits were purchased from Pierce Chemical Co. (Rockford, IL). Phosphospecific antibodies specific for DARPP-32 T75, DARPP-32 S137 and spinophilin S94 were kindly provided by Dr. Paul Greengard (Rockefeller University). Anti-phospho-T34 DARPP-32 antibodies were provided by Dr. Angus Nairn (Rockefeller University). Anti-phospho-S845 GluR1, phospho-S831 GluR1, phospho-S897 NR1, and phospho-S133 CREB antibodies were obtained from Upstate USA, Inc. (Charlottesville, VA). Alexa-680 fluorescent labeled goat anti-mouse IgG was obtained from Molecular Probes (Eugene, OR). IR dye 800CW fluorescent tag labeled goat anti-rabbit IgG was purchased from Rockland Immunochemicals (Gilbertsville, PA). Blocking buffer for Western blotting was obtained from LiCor (Lincoln, NE).

Animal experimental procedures: Rats were divided into 5 groups with 6 animals in each group. Group one was injected subcutaneously (sc) with the vehicle saline (0.5 ml/kg) to serve as controls. Group two was injected with sarin at dose of 1.0 x $LD_{50}$ ($LD_{50}$ = 125 ug/kg, sc) and euthanized 15 min after injection. Group three was injected with sarin at dose of 1.0 x $LD_{50}$ and euthanized 30 min after injection. Group four was injected with sarin at dose of 0.5 x $LD_{50}$ and euthanized 15 min after injection. Group five was injected with sarin at dose of 0.5 x $LD_{50}$ and euthanized 30 min after injection. Animals were euthanized by a head-focused microwave device (3.0 kW, 2.45 MHz for 1.0 sec/100 gram body weight; Gerling-Moore Metabostat System, Gerling-Moore, Inc., Santa Clara, CA) to arrest alterations of phosphorylation state *in vivo* at specified times after injection. Because of the rapid response observed in the slices preparation, 15 and 30 min after sarin administration were investigated in this study. Cerebral cortex, striatum and hippocampus were dissected rapidly after the microwave procedure and stored at – 80° C until phosphorylation analysis.

Sample Processing: Frozen tissue samples from microwaved animals were sonicated in 1% sodium dodecyl sulfate (SDS) and boiled for 10 min. Small aliquots of the homogenate were retained for protein determination by the bicinchoninic acid (BCA) protein assay method (Pierce

Chemical Co., Rockford, IL). Equal amounts of protein were processed using 10% acrylamide gels as described by Nishi et al. (1997) and immunoblotted as described below.

DARPP-32 phosphorylation sites were analyzed by the use of phospho-specific antibodies that have been developed to specifically monitor changes in phosphorylation by the procedure described by Czernick et al., 1991. Phosphorylation sites examined were the T34, T75, S102 and S137 of DARPP-32, Ser-94 (S94) of spinophilin, Ser-897 of the NMDA receptor NR1 subunit, and Ser-845 (S845) of the AMPA receptor GluR1 subunit (see Table 1). The brain region examined was initially the striatum.

Immunoblotting for DARPP-32 phosphorylated at T34, T75 or S137: Aliquots (3 µl) of the striatal homogenate were used for protein determination. Equal amounts of protein (50 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (BioRad, Hercules, CA). The membranes were blocked in Tris-buffered saline (TBS)/Tween with LiCor Blocking Buffer (LiCor, Lincoln NE) followed by incubation with antibodies against phospho[T34]-DARPP-32, phospho[T75]-DARPP-32, phospho[S137]-DARPP-32 or total DARPP-32. The membranes were then washed 4 times for 5 min each with TBS/Tween and antibody binding revealed using Alexa 680 labeled goat anti-mouse IgG (Molecular Probes, Eugene OR) or IRdye 800CW labeled goat anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA). Antibody binding was detected and quantitated using a LiCor Odyssey infrared fluorescent detection system (LiCor, Lincoln, NE).

Reagents for detecting the other phosphorylation sites of interest, including S133 CREB, T183 ERK, S897 NR1, S831 and S845 GluR1, and S94 spinophilin, have been previously described (Svenningsson et al., 2002; Pozzi et al., 2003).

Data Analysis: The state of phosphorylation of several neuronally enriched phosphoproteins was monitored and quantified in striatal samples from sarin-exposed rats and saline- treated control rats at 15 and 30 min after sarin or saline administration. These sites and their description are listed in Table 1. The levels for each phosphoprotein site were averaged across all animals in the group (N=5 or 6). Levels of phosphorylation at each site were quantified and expressed as a percent $\pm$ SEM of levels present in striatum of the saline-injected control rats. Levels of phosphorylation at each site were compared between the two sarin-treated groups and the saline (control) group. Statistical analyses were performed using paired Student's t-test or ANOVA with Newman-Keuls post-hoc test. A difference of $p<0.05$ was considered significant.

## RESULTS

Behavioral Observations: Rats were injected (sc) with either a 0.5 x $LD_{50}$ or a 1.0 x $LD_{50}$ dose of sarin ($LD_{50}$ = 125 µg/kg, sc). These rats, and saline-injected control animals, were euthanized by focused microwave irradiation of the head either 15 min or 30 min later. All but one rat administered the 1.0 x $LD_{50}$ dose of sarin showed behavioral signs of seizure activity and/or convulsions. The rat was excluded from further analysis. None of the rats administered a 0.5 x $LD_{50}$ dose of sarin displayed symptoms of seizure activity.

Biochemical Observations: Three brain regions, including striatum, cortex, and hippocampus, were dissected from the brains of all treated rats. These brain samples were transported on dry ice to ITI for analysis. Preliminary analysis has been performed on the striatal samples from sarin-treated rats and their saline-injected controls. Analysis on cortical and hippocampal samples was not yet done. The data are summarized in Table 2.

Rats administered a 0.5 x $LD_{50}$ dose (62.5 µg/kg, sc) of sarin displayed a small but significant increase in the state of phosphorylation of DARPP-32 at T75 30 min after administration (Table 2, $p=0.03$, t-test). The state of phosphorylation of the other proteins measured was not significantly changed after exposure to sarin at this dose level at either time point examined.

Rats exposed to a 1.0 x $LD_{50}$ dose (125 µg/kg, s.c.) of sarin displayed significant changes in phosphorylation of several sites. Three sites increased in phosphorylation level 15 min after sarin treatment (Figure 2): S133 of CREB, T183 of ERK, and T75 of DARPP-32. Thus, levels of T75-phosphorylated DARPP-32 were increased in both symptomatic and asymptomatic rats. The state of phosphorylation of several sites was reduced 30 min (but not 15 min) after 1.0 x $LD_{50}$ sarin treatment: T34, S102, and S137 of DARPP-32, S94 of Spinophilin, and S897 of NR1. The reduction in phosphorylation at S897 of NR1 was not indicative of a generalized dephosphorylation of all glutamate receptors since levels of phosphorylation of S845 of the AMPA receptor subunit GluR1 were unaffected by 1.0 x $LD_{50}$ sarin treatment.

In summary, rats receiving a sub-threshold dose of sarin (i.e., 0.5 x $LD_{50}$) were asymptomatic for seizures. A selective increase in phospho-T75 DARPP-32 levels was observed in striatum from these rats 30 min after exposure. Rats displaying seizure activity following administration of a 1.0 x $LD_{50}$ dose of sarin displayed changes in several phosphoproteins, including T75 DARPP-32. Transient increases in T75 DARPP-32, T183 ERK, and S133 CREB phosphorylation at 15 min were followed by reductions in phosphorylation at three DARPP-32 sites (T34, S102, and S137) as well as reductions in S94 spinophilin and S897 NR1 at 30 min. Another glutamate receptor site, S845 GluR1, was unaffected by sarin treatment.

## SIGNIFICANCE OF FINDINGS

The results of the present study serve to identify unique patterns of protein phosphorylation changes in the striatum that are associated with exposure of rats to either seizure-inducing or sub-threshold doses of the nerve agent sarin. Consistent with previous studies (Hulet et al., 2002; Scremin et al., 2003), rats receiving a high dose of sarin (i.e., 1.0 x $LD_{50}$ dose) showed characteristic signs of seizure activity and convulsions. Rats treated with a 1.0 x $LD_{50}$ dose of sarin displayed rapid (i.e., within 15 min) and transient increases in the state of phosphorylation of the protein kinase ERK and one of its major substrates--the transcription factor CREB in striatum. Since CREB is a key regulator of gene transcription in the brain, these data indicate that an early response to sarin exposure is the activation of signaling pathways that regulate gene expression. In addition, this high dose of sarin also increased the phosphorylation state of DARPP-32 at a site (T75) that, when phosphorylated by the cyclin-dependent kinase 5 (CDK5), converts the phosphoprotein into an inhibitor of PKA (see Figure 1), a major protein kinase. PKA is a key component responsible for mediating the effects of activation of the D1-subclass of

dopamine (DA) receptors in striatum. This role of PKA in DA signaling is mediated, in part, via the PKA-dependent phosphorylation of DARPP-32 at a site (T34) that converts the phosphoprotein into a potent inhibitor of PP-1, a major serine/threonine phosphatase that controls the state of phosphorylation and activity of many neuronal proteins. Thus, sarin, by increasing levels of T75-phosphorylated DARPP-32, would be expected to suppress striatal signaling via D1-subtype DA receptors. This increase in phospho[T75]-DARPP-32 level, and the expected attenuation of PKA activity in striatum, may be responsible for the subsequent reduction at 30 min in the phosphorylation state of three PKA substrates, including T34 DARPP-32, spinophilin (at S94) and NR1 (at S897). Phosphorylation of spinophilin, a PP-1 targeting protein, at S94 alters the ability of this PP-1 targeting protein to interact with the actin cytoskeleton and, presumably, to target PP-1 to certain neuronal substrates, including NMDA receptors. The reduction in phosphorylation of the NMDA receptor subunit NR1 at S897 would be expected to reduce NMDA receptor currents. Interestingly, whereas sarin exposure profoundly reduced phosphorylation of the NMDA receptor, it had no effect on the phosphorylation state of the AMPA-type glutamate receptor subunit GluR1, which suggests that the nerve agent has a selective impact on NMDA receptor activity.

However, some of the above results would not be predicted from previous work. As stated above, treatment of neostriatal slices with nicotine (100 μM), a cholinergic acetylcholine receptor agonist, stimulated DARPP-32 phosphorylation on T34 by approximately 7-fold at 15 and 30 seconds of incubation. This effect of nicotine (100 μM) on T34 phosphorylation was abolished by the dopamine D1 antagonist SCH23390. A seizure-inducing dose of sarin would be expected to result in high levels of acetylcholine, the endogenous agonist that acts at both muscarinic and nicotinic receptors, and such cholinergic stimulation leads to increased dopamine activity as reflected by increases in dopamine metabolites seen in striatum following convulsant doses of nerve agent (Shih, 1982; Shih and McDonough, 1997). That dopamine is involved in the etiology of nerve agent-induced seizures is also supported by the finding that the D1 antagonist SCH23390 will also block seizure activity produced by the nerve agent soman (Bourne et al., 2001). Given the above, it could be reasoned that a seizure-producing dose of the nerve agent sarin would increase DARPP-32 phosphorylation on T34. Yet T34 DARPP-32 phosphorylation actually showed a significant decrease 30 min after sarin exposure, a time when seizures were observed.

In contrast to the seizure-inducing effects of a 1.0 x $LD_{50}$ dose of sarin, a dose of 0.5 x $LD_{50}$ sarin was not observed to induce symptoms of seizure activity in rats. Despite being asymptomatic for sarin-induced seizures, the rats displayed a significant and selective increase in phospho[T75]-DARPP-32 levels in striatum, which was observed 30 min after sarin exposure. The appearance of increases in phospho[T75]-DARPP-32 levels at doses of sarin that are sub-threshold for seizure induction suggests that the signaling pathways that control phosphorylation of this site (e.g., CDK5) may be early events in the brain response to nerve agents. Since CDK5 has been associated with normal neuronal development and with the structural reorganization of neurons in response to drugs of abuse, it may be an excellent candidate for mediating the subtle, long-lasting perceptual and motor deficits associated with low-level sarin exposure.

These studies demonstrate that phosphorylation of specific phosphoproteins is a sensitive procedure to monitor the effects of nerve agent exposure *in vivo*. Further work will be performed to compare the effects in different brain regions and/or with additional nerve agents. This effort provides an excellent start to the search for inhibitors of these cellular pathways that could serve as novel antidotes to nerve agent exposure.

Table 1. Phosphorylation sites examined and their functions

| **Phospho-Site Abbreviation** | **Identity/Description of Site** |
|---|---|
| T75 | Thr75 is a CDK5-dependent site on DARPP-32 controlling the PKA-inhibitor activity of DARPP-32 |
| S133 | Ser133 on CREB and is essential for regulating CREB function |
| T183 | Thr183 on ERK (both the 42kDa and 44kDa forms are measured) |
| T34 | Thr34 is a PKA-dependent site on DARPP-32 that converts DARPP-32 into a PP-1 inhibitor |
| S102 | Ser102 is a CK2-dependent site on DARPP-32 that enhances T34 phosphorylation |
| S94 | Ser94 is a PKA-dependent site on spinophilin controlling association of this PP-1 targeting protein with actin |
| S137 | Ser137 is a CK1 site on DARPP-32 that also facilitates Thr34 phosphorylation |
| S897 | Ser897 is a PKA-dependent site on the NR1 subunit of the NMDA receptors that is involved in regulating NMDA receptor conductance |
| S845 | Ser845 is a PKA-dependent site on the GluR1 subunit of the AMPA receptors that enhances open-time probability of the receptor channel |

Footnote:

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CDK5 = Cyclin-dependent kinase 5
CK1 = casein kinase I
CK2 = casein kinase II
CREB = cyclic AMP response element binding protein
DARPP-32 = dopamine (DA) and cAMP-regulated phosphoprotein of molecular weight 32KDa
ERK = extracellular signal-regulated protein kinase
GluR1 = subunit 1 of AMPA-responsive type glutamate receptor
NMDA = N-methyl-D-aspartate
NR1 = N-methyl-D-aspartate receptor subunit 1
PKA = cAMP-dependent protein kinase
PP-1 = protein phosphatase 1

Table 2. Effect of a 0.5 x $LD_{50}$ dose of sarin on the state of phosphorylation of several striatal phosphoproteins. Levels of phosphorylation at each site are quantified and expressed as a percent ± SEM of levels present in striatum of the saline-injected control rats. Data were analyzed by Student's t-test.

| Phospho-Site | Control ± SEM % | 0.5 $LD_{50}$ (15 min) ± SEM % of control | 0.5 $LD_{50}$ (30 min) ± SEM % of control | P value |
|---|---|---|---|---|
| T75 | 100 ± 11.9 | 101.2 ± 9.1 | 129.8 ± 8.8 * | *P=0.03 vs. Control |
| S133 | 100 ± 5.6 | 90.8 ± 8.8 | 95.2 ± 12.8 | NS |
| T183 | 100 ± 6.9 | 78.3 ± 9.2 | 90.3 ± 6.3 | NS |
| T34 | 100 ± 13.7 | 87.1 ± 22.7 | 130.6 ± 14.6 | NS |
| S102 | 100 ± 5.2 | 109.8 ± 4.5 | 116 ± 9.3 | NS |
| S94 | 100 ± 17.3 | 93.9 ± 24.2 | 95.2 ± 12.8 | NS |
| S137 | 100 ± 6.9 | 95.3 ± 11.2 | 116.4 ± 19.7 | NS |
| S897 | 100 ± 13.9 | 110 ± 15.9 | 104.7 ± 4.6 | NS |
| S845 | 100 ± 5.5 | 94.2 ± 17.0 | 100.2±16.04 | NS |

NS = Not significant

Figure 1. Phosphorylation sites on DARPP-32.

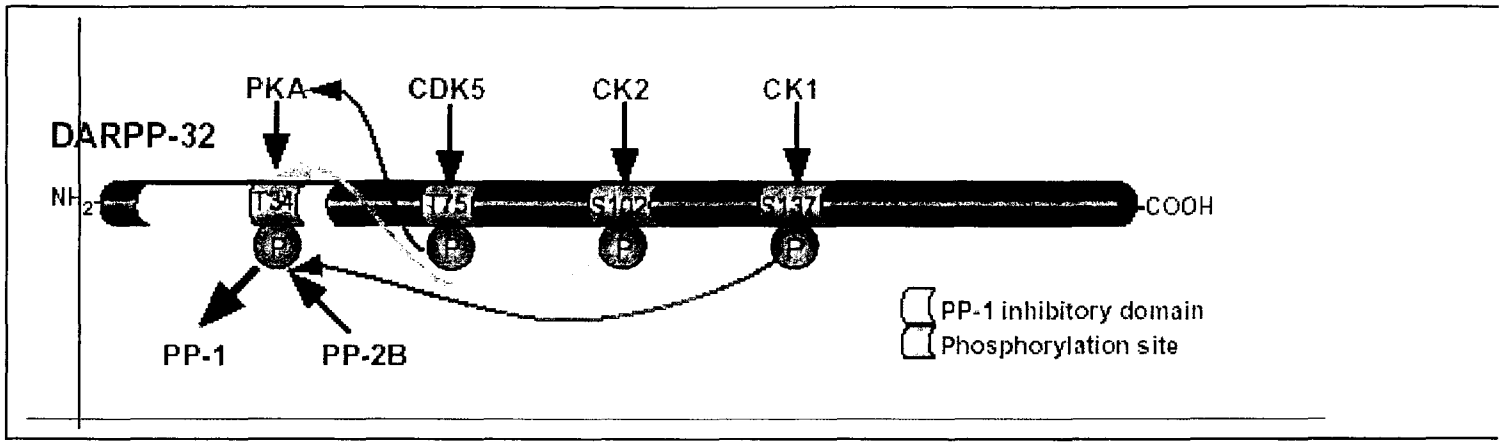
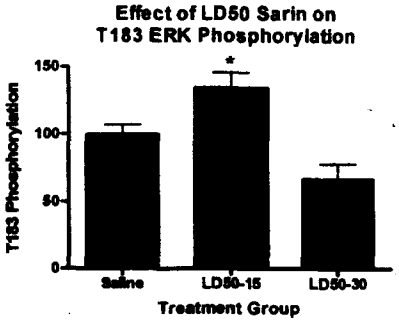


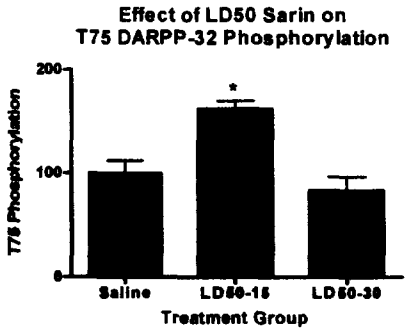

**Figure 1.** Diagram showing sites of phosphorylation of DARPP-32 and indicating the effect of each phosphorylation site on DARPP-32 function. Phosphorylation of DARPP-32 at T34 by PKA leads to inhibition of PP1 activity, whereas phosphorylation of DARPP-32 at T75 by CDK5 leads to inhibition of PKA activity (dark arrow). Phosphorylation of DARPP-32 at S102 by CK2 promotes phosphorylation at T34 by PKA (light arrow). Phosphorylation of DARPP-32 at S137 promotes phosphorylation at T34 (dark arrow) by reducing dephosphorylation at this site by PP-2B. See text for details.

**Figure 2.** The effects of 1.0 x $LD_{50}$ sarin on the state of phosphorylation of striatal phosphoproteins. Rats were treated with 1.0 x $LD_{50}$ sarin (125 ug/kg, sc) and euthanized at either 15 min (LD50-15) or 30 min (LD50-30) by head-focused microwaved irradiation and phosphoproteins analysed in the striatum. Levels of phosphorylation at each site were quantified and expressed as a percent $\pm$ SEM of levels present in striatum of the saline-injected control rats. Levels of phosphorylation at each site were compared between the two sarin-treated groups and the saline (control) group. Statistical analyses were performed using ANOVA with Newman-Keuls post-hoc test or paired Student's t-test. A difference of $p<.05$ was considered significant. *$P<.05$ vs. saline group; #$P<.05$ vs.LD50-15 group.

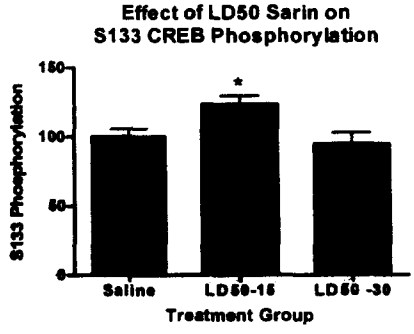
# Figure 2. Effect of 1LD50 Sarin on the State of Phosphorylation of Striatal Phosphoproteins



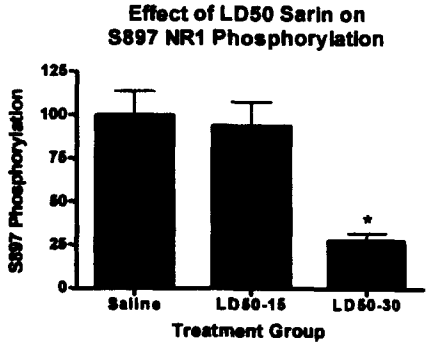

*vs. Saline or LD50-30 by ANOVA with Newman-Keuls Post-Hoc test



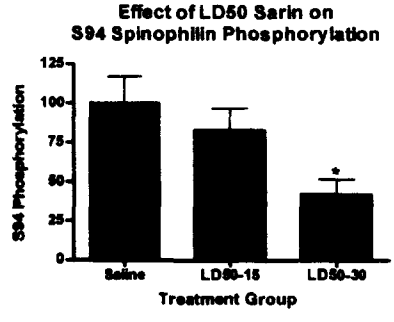

* vs Saline and LD50-30 by ANOVA with Newman-Keuls Post-Hoc test



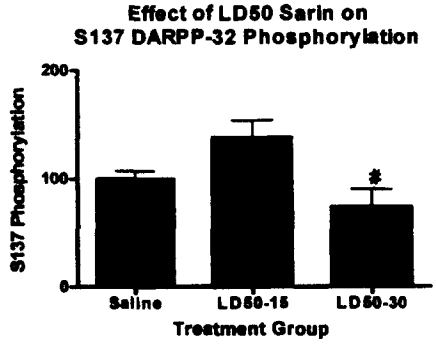

*vs. Saline by ANOVA with Newman-Keuls Post-Hoc test



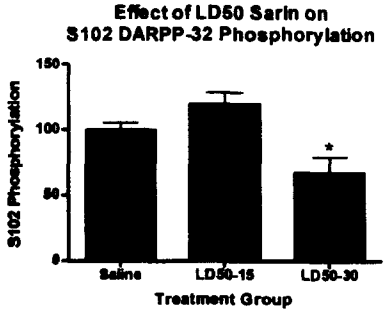

*vs. Saline and LD50-15 by ANOVA with Newman-Keuls Post-Hoc test



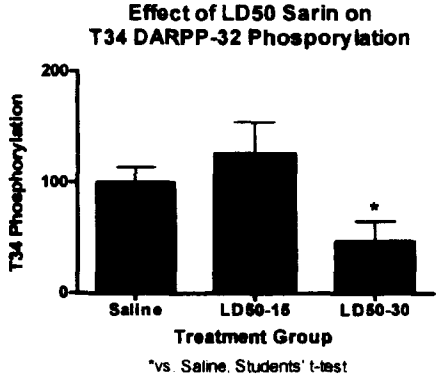

* vs Saline by ANOVA with Newman-Keuls Post-Hoc test



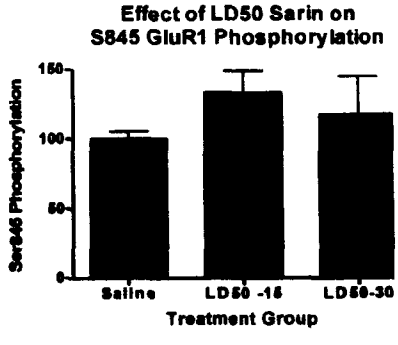

# vs. LD50-15 by ANOVA with Newman-Keuls Post-Hoc test




*vs. Saline or LD50-15 by ANOVA with Newman-Keuls Post Hoc-test




*vs. Saline, Students' t-test



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## ABBREVIATIONS

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BCA = bicinchoninic acid
cAMP= cyclic adenosine monophosphate
CCK = cholecystokinin
CDK5 = cyclin-dependent kinase 5
CK1 = casein kinase I
CK2 = casein kinase II
cGMP = cyclic guanidine monophosphate
CREB = cyclic AMP response element binding protein
DA = dopamine
DARPP-32 = dopamine (DA) and cAMP-regulated phosphoprotein of molecular weight 32KDa
ECL = enhanced chemiluminescence
ERK = extracellular signal-regulated protein kinase
GABA = γ-aminobutyric acid
GluR1 = subunit 1 of AMPA-responsive type glutamate receptor
$LD_{50}$ = median lethal dose or lethal dose 50%
NMDA = N-methyl-D-aspartate
NR1 = subunit 1 of N-methyl-D-aspartate-responsive type glutamate receptor
PKA = cAMP-dependent protein kinase
PKG = cGMP-dependent protein kinase
PP-1 = protein phosphatase 1
PP-2B = protein phosphatase 2B
PVDF = polyvinylidene fluoride
S94 = serine residue 94 on spinophilin
S102 = serine residue 102 on DARPP-32
S133 = serine residue 133 on CREB
S137 = serine residue 137 on DARP-P32
S845 = serine residue 845 on the AMPA receptor GluR1 subunit
S897 = serine residue 897 on the NMDA receptor NR1 subunit
SDS = sodium dodecyl sulfate
SDS-PAGE = SDS-polyacrylamide gel electrophoresis
T34 = threonine residue 34 on DARPP-32
T75 = threonine residue 75 on DARPP-32
T183 = threonine residue 183 on ERK
TBS = Tris-buffered saline